oligonucleotides encoding both the murine and the human amino acids at seven V_H and one V_K framework position as selected above were synthesized (Fig. 1 residues marked with asterisk). All of these sites were characterized by synthesizing a combinatorial library that expressed all possible combinations of the murine and human amino acids found at these residues. The total diversity of this library, termed Hu I, was 2^8 or 256 variants (Table I).

The Hu I combinatorial library was first screened by an ELISA that permits the rapid assessment of the relative affinities of the variants (Watkins et. al., (1997)). Briefly, microtiter plates were coated with 5 μg/ml goat anti-human kappa (Southern Biotechnology) and blocked with 3% BSA in PBS. Certain Fabs were cloned into an expression vector under the control of the arabinose-regulated BAD promoter. In addition, a six-histidine tag was fused to the carboxyl-terminus of the H chain to permit purification with nickel-chelating resins. Purified Fab was quantitated as described (Watkins et. al., 1997). Next, 50 μl Fab from the *Escherichia coli* culture supernatant or from the cell lysate, was incubated with the plate 1 h at 25°C, the plate was washed three times with PBS containing 0.1% Tween 20, and incubated with 0.1 μg/ml CD40-Ig in PBS containing 1% BSA for 2 h at 25°C. The plate was washed three times with PBS containing 0.1% Tween 20 and goat anti-mouse IgG_{2b}-alkaline phosphatase conjugate diluted 3000-fold in PBS containing 1% BSA was added for 1 h at 25°C. The plate was washed three times with PBS containing 0.1% Tween 20 and was developed as described (Watkins et. al., (1997)).

Although variants that bind the target antigen with affinities comparable to, or better than the

chimeric Fab were identified, the majority of Hu I clones screened were less active than the chimeric anti-CD40 Fab. Approximately 6% of randomly selected Hu I variants displayed binding activities comparable to the chimeric Fab (data not shown). The identification of multiple Hu I variants with activity comparable to the chimeric CD40 is consistent with the interpretation that the most critical framework residues were included in the combinatorial library.

The kinetic constants for the interaction between CD40 and the anti-CD40 variants were determined by surface plasmon resonance (BIAcore). CD40-Ig fusion protein was immobilized to a (1-ethyl-3-[3-dimethylaminopropyl]-carbodiimide hydrochloride) and *N*-hydroxysuccinimide-activated sensor chip CM5 by injecting 8 μl of 10 μg/ml CD40-Ig in 10 mM sodium acetate, pH 4. CD40-Ig was immobilized at a low density (~150 RU) to prevent rebinding of Fabs during the dissociation phase. To obtain association rate constants (k_{on}), the binding rate at six different Fab concentrations ranging from 25-600 nM in PBS was determined at a flow rate of 20 μl/min. Dissociation rate constants (k_{off}) were the average of six measurements obtained by analyzing the dissociation phase. Sensorgrams were analyzed with the BIAevaluation 3.0 program. K_d was calculated from $K_d = k_{off}/k_{on}$. Residual Fab was removed after each measurement by prolonged dissociation.

Figure. 2A shows bacterially-expressed chimeric anti-CD40 Fab and certain variants from each of the libraries were titrated on immobilized antigen. Chimeric (filled circles), Hu I-19C11 (open circles), Hu II-CW43 (open squares), Hu III-2B8 (filled triangles), and an

irrelevant (filled squares) Fab were released from the periplasmic space of 15 ml bacterial cultures and serial dilutions were incubated with CD40-Ig antigen immobilized on microtiter plates. See below for description of HuII and HuIII libraries. Antibody binding was quantitated as described above. These measurements confirm the identification of multiple variants with enhanced affinity. For example, clone 19C11 binds the CD40 receptor with higher affinity than the chimeric Fab, as demonstrated by the shift in the titration profile (compare open circles with filled circles). DNA sequencing of 34 of the most active clones led to the identification of 24 unique framework combinations, each containing 2-6 murine framework residues (data not shown).

LCDR3 and HCDR3 contact antigen directly, interact with the other CDRs, and often affect the affinity and specificity of antibodies significantly (Wilson & Stanfield, (1993); Padlan, (1994)). In addition, the conformation of LCDR3 and HCDR3 are determined in part by certain framework residues. Therefore, to identify the most active antibody it could be beneficial to construct combinatorial libraries that optimize the third CDR of the H and L chains in conjunction with selecting the most active framework.

Codon-based mutagenesis (Glaser et. al., (1992)) was used to synthesize oligonucleotides that introduce mutations at every position in HCDR3, one at a time, resulting in the expression of all 20 amino acids at each CDR residue from Ser^{95}-Tyr^{102} (Fig. 1, underlined). Briefly, for library construction, the overlapping oligonucleotides encoding the framework library and non-library murine CDR were combined with 25 pmol of the